Live Attenuated Listeria Monocytogenes Effectively Treats Hepatic Colorectal Cancer Metastases and Is Strongly Enhanced by Depletion of Regulatory T Cells

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Abstract

The liver represents a major and frequently sole site of metastases for many types of cancer, particularly gastrointestinal cancers. We showed previously that coadministration of an engineered hepatic-targeting Listeria monocytogenes (LM) with a cancer vaccine enhanced the antitumor effect of vaccine-induced T cells selectively against hepatic metastases. Here, we show that administration of multiple doses of LM, in the absence of vaccine, generates therapeutic responses against hepatic metastases. LM treatment of mice bearing hepatic metastases induced tumor-specific CD8+ T-cell responses that were enhanced by depletion of regulatory T (Treg) cells by either anti-CD25 or cyclophosphamide treatment. Antitumor activity of LM further depended on natural killer (NK) cell activation but was inhibited by presence of a subset of NK T cells. These results show the utility of LM in the treatment of hepatic metastases even in the absence of vaccine administration and further suggest that blockade of Treg cells and NK T cells will enhance antitumor activity.

Introduction

It is now well established that biological characteristics of cancer metastases depend significantly on the specific organ in which they are growing. Likewise, therapeutic responses differ according to the site of metastasis. Many mechanical approaches for organ-specific delivery of therapeutics, such as isolating the vascular bed (1, 2), have been explored because individual organs are frequently the primary or sole sites of metastatic disease (3). The liver is a commonly involved organ for metastasis, particularly in gastrointestinal malignancies (4).

We reported recently the application of an engineered microbe, Listeria monocytogenes (LM), to target immune responses selectively into the liver (5). LM primarily infects the liver whether administered orally or parenterally (6). Wild-type (WT) LM infects both hepatocytes and hematopoietically derived cells within the liver, inducing very potent innate immune responses as well as T-cell responses against its own antigens. The potent immunogenicity of LM makes it a promising vaccine vector (7). Importantly, disruption of two LM genes, actin A (actA) and internalin B (InlB), resulted in a highly virulence-attenuated strain (LD50 4–5 logs higher than WT LM) that retained full capacity to induce intrahepatic immune responses (8–12). We found that a single dose of an attenuated LM strain enhanced antitumor activity of a granulocyte macrophage colony-stimulating factor (GM-CSF)–transduced tumor vaccine against hepatic metastases but not lung metastases, showing that LM selectively targeted vaccine-induced immunity to the liver (5). We postulated that this hepatic targeting resulted from the innate immune responses to the LM in the liver that attracted vaccine-induced, tumor-specific T cells. This hypothesis was supported by the finding of increased numbers of tumor-specific CD8+ T cells in the livers of tumor-bearing animals treated with both vaccine and LM.

In the current work, we examined the capacity of LM to induce antitumor responses against liver metastases in the absence of a specific vaccine. We found that multiple doses of a virulence-attenuated LM administered without vaccination induced antitumor responses that depended on CD8+ T cells. We further found that the antitumor responses could be enhanced significantly by elimination or inhibition of regulatory T (Treg) cells. Surprisingly, although natural killer (NK) cells were critical for antitumor responses, antitumor responses were enhanced in NK T cell–deficient mice. These findings support the application of virulence-attenuated LM in the treatment of hepatic metastases and further suggest that inhibition of Treg cells and NK T cells may enhance this effect.

Materials and Methods

Animals and tumor cell lines. BALB/c mice (8–10 weeks old, female) were purchased from the National Cancer Institute and CD1d knockout (CD1d KO) mice were from Jackson River Laboratory. Colon tumor 26 cells (CT26) are murine colon adenocarcinoma cells derived from BALB/c mice (13). The cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (HyClone), 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, nonessential amino acids (1% of 100× stock), 25 mmol/L HEPES buffer, and 50 µmol/L 2-ME (C-Media). All experimental subjects were treated ethically in accordance with a protocol approved by the Johns Hopkins Animal Care and Use Committee.

LM and cyclophosphamide. LM actA-deleted and InlB-deleted strain (LM-D) was made by Cerus Corp. (also known as CRS-100). LM-actA was derived from WT LM (LM-Wild) and contains in-frame deletions in the actA (9). LM strains were grown in Brain Heart Infusion (Difco Laboratories) medium. Bacteria for animal studies were harvested at midlog phase of growth, purified by standard methods, formulated in PBS/8% DMSO at a concentration of ~1 × 1010 colony-forming units (CFU)/mL, and stored at −80°C. For injection, bacteria were thawed on ice and diluted.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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in PBS according to injection doses in a volume of 100 µL corresponding to 0.1 median lethality (0.1 × LD₅₀) in BALB/c mice, as described (8). Cyclophosphamide was purchased from Sigma.

**Murine hepatic metastasis model.** Mice were given isolated hepatic metastases using a hemispleen injection technique (5, 14).

**Treatment of mice with LM and cyclophosphamide in the tumor model.** Mice that received LM-D treatment received i.p. injections of 0.1 × LD₅₀ (1 × 10⁴ CFU). Depending on the experiment, they received one or three doses separated by 3 days. Also depending on the experiment, the injections were initiated on day 3 or day 5. Cyclophosphamide treatment was administrated on day 4 in BALB/c mice, using a single i.p. injection of 1.6 mg (100 mg/kg) of cyclophosphamide in 100 µL PBS. Cyclophosphamide was only given once and, when used, was given the day before initiation of LM-D treatment.

**Rechallenge of mice for in vivo assessment of memory response.** Mice were challenged with hepatic metastases and treated with LM-D and cyclophosphamide. Forty days after tumor injection, the surviving mice and naïve BALB/c mice were rechallenged s.c. with 1 × 10⁵ CT26 cells suspended in 100 µL PBS using a 27-gauge needle into the right abdome nal wall. Tumor volumes were measured in cubic millimeter with calipers and calculated with the following formula: a × b² / 2, where a is the larger and b is the smaller of the two dimensions.

**Isolation and analysis of liver-infiltrating lymphocytes.** For analysis of CD4⁺ and CD8⁺ T cells, three livers were processed per group and pooled. Each liver was mashed through a 100-µm nylon mesh and brought to a volume of 45 ml medium. This suspension was spun at 1,500 rpm for 10 min at 4°C. The cell pellets were resuspended in 5 ml of 10% Percoll, 10 ml of RPMI 1640, and 2 to 3 drops of heparin and vortexed and centrifuged at room temperature for 20 min, without brake. Supernatants were aspirated. Pellets were resuspended in 5 ml C-Media.

For analysis of AH1-specific CD8⁺ T cells, the cells were enriched for magnetic CD8⁺ T-cell isolation protocol (MACS, Miltenyi Biotech). These cells were then assayed for presence of AH1-specific T-cell receptors (TCR) as described below.

**Cell staining and flow cytometry.** Following the isolation of liver-infiltrating immune cell populations from the mouse livers, cells were stained with CD4-FITC (Caltag), B220-FITC (PharMingen), CD8-chromey (PharMingen), and CD25-APC (PharMingen) and assayed on a FACScan flow cytometer (Becton Dickinson). Analysis of AH1 tumor-specific CD8⁺ T cells was performed using Ld tetramer loaded with either AH1 (SPSVYH9KF) or the negative control β-galactosidase (TPHPARIGL) provided by the NIH core facility.

**Intracellular staining for FoxP3 and flow cytometry.** After staining with CD4 and CD25 following the protocol, cells were resuspended by 1 ml cold Fix/Perm buffer (eBioscience) and incubated at 4°C overnight in the dark. The cells were washed with permeabilization buffer and blocked with FcγRII/III antibody (PharMingen) for 20 min, followed by the addition of anti-mouse FoxP3 (FJK-16c eBioscience) antibody, and incubated for 45 min. Cells were washed with permeabilization buffer and assayed on a FACScan flow cytometer (BD Biosciences).

**Intracellular staining for IFN-γ and flow cytometry.** The harvested cells were resuspended in C-Media. Golgistop (1:1,000; BD Biosciences) and AH1 peptide (10 µg/ml) were added and incubated at 37°C for 5 h. After washing, these cells were stained with CD8 following the procedure described above. The cells were stained with Fix/Perm buffer (eBioscience) and incubated at 4°C overnight in the dark. The cells were washed with permeabilization buffer and incubated with FcγRII/III antibody (PharMingen) for 20 min, followed by the addition of anti-mouse FoxP3 (FJK-16c eBioscience) antibody, and incubated for 45 min. Cells were washed with permeabilization buffer and assayed on a FACScan flow cytometer (BD Biosciences).

**LM and Immune Manipulation Treat Hepatic Metastases**

**Results**

*LM* has antitumor activity against hepatic metastases. We compared the efficacy of single or multiple i.p. injections of a strain of *LM* that was genetically attenuated by eliminating expression of actA and InlB genes (*LM-D*; ref. 8) against no treatment in a hepatic metastasis model. Figure 1A shows that LM-D treatment resulted in increased survival of mice with hepatic metastases. In three replicative experiments, treatment with three injections 3 days apart beginning on day 3 (LM-D3) provided the greatest survival advantage (44%; *P* < 0.001 versus no treatment). Treatment with a single injection on day 3 (LM-D1) provided some but less survival advantage (20%; *n* = 9–10; *P* < 0.001 versus no treatment).

**Role of T-cell subsets in the treatment effect of LM-D against hepatic metastases.** In *in vivo* antibody depletion experiments were done. Tumor-bearing mice given with LM-D3 treatment were also given depleting antibodies before tumor challenge in the hepatic metastasis model. As before, mice given LM-D3 treatment had a 44% survival rate (LM-D3 versus no treatment; *P* < 0.001). This survival effect, however, was abrogated when animals were depleted of CD8⁺ T cells, showing that the antitumor effect was dependent on adaptive immunity (Fig. 1B). Mice depleted of CD4⁺ T cells seemed to still have some survival benefit to the LM-D3 treatment (although less so). This result does not absolutely eliminate a role of CD4⁺ T cells because it is now appreciated that the CD4⁺ subset contains both helper T cells and Treg cells. Apparently, depletion of CD4⁺ T cells resulted in offsetting responses from elimination of both helper T cells and Treg cells. Therefore, CD25 depletion was carried out as a means of eliminating Treg cells. Although CD25 is not Treg specific, the Treg population is generally enriched in CD25⁺ cells (16). Mice depleted of CD25⁺ cells while receiving LM-D3 treatment showed significantly enhanced antitumor efficacy (89% survival) compared with the mice treated with LM-D3 with no depletion (*P* = 0.035; Fig. 1B). These results confirm that CD8⁺ T-cell populations are critical for LM-D–mediated antitumor responses, whereas a subset of CD25⁺ T cells impede the intrahepatic antitumor response (n = 8–9).

**Role of NK and NK T cells in the antitumor effect of LM-D.** Two related populations of potential effector cells that are enriched in the liver are NK cells and NK T cells. Both are considered part
of the innate immune system despite the expression of a TCR by NK T cells. The TCR expressed by most NK T cells is invariant and recognizes self-derived and bacterial-derived glycolipids presented by CD1d (17). Anti–asialo GM1 antibodies selectively deplete NK cells, whereas CD1d KO mice are selectively depleted of NK T cells, whereas the NK cell compartment is intact (18). All mice depleted with anti-asialo GM1 antibody, challenged with tumor, and then treated with LM-D3 died due to tumor progression 20 days earlier than the group with no depletion, suggesting a strong contribution of the NK population toward slowing tumor growth. To study the contribution of NK T cells to the antitumor effect in this model, CD1d KO BALB/c mice were challenged with tumor, treated with LM-D3 therapy, and compared with WT BALB/c undergoing similar treatment (n = 7–10; Fig. 1C). Surprisingly, CD1d KO mice showed significantly enhanced survival relative to WT BALB/c mice (CD1d KO with LM-D3, 88% survival; WT with LM-D3, 38% survival; P = 0.044). These results suggest that whereas NK cells provide a strong positive antitumor effect, a subset of NK T cells inhibit antitumor immunity with this treatment approach.

**Tumor-specific CD8+ T-cell responses in tumor-bearing livers as a result of LM-D3 treatment.** The identification of an immunodominant MHC class I (Ld) restricted target from CT26 (AH1) allowed us to use Ld-AH1 tetramers to directly assess the numbers of AH1-specific T cells within the liver (19, 20). Compared with untreated animals, a significant percentage of AH1-specific T cells (3.1% versus 1.4% of intrahepatic CD8+ T cells) could be detected in livers from tumor-bearing mice treated with LM-D3 on day 14 (Fig. 2A). This increase was detected in the lymphocytes from liver, whereas the lymphocytes from spleen did not show any increase (data not shown). Additionally, the total number of lymphocytes in the livers of mice was highest in the mice given with LM-D3 treatment on days 10 and 14 (Fig. 2B). These results are in concordance with the CD8+ T-cell depletion experiments described in Fig. 1B and highlight the liver-specific targeting role of LM-D3 treatment.

**The combination of LM-D3 treatment and cyclophosphamide enhances survival in the liver metastasis model.** Two results suggested that Treg cells may have played a significant role in inhibiting the antitumor responses induced by LM-D3 treatment against liver metastases. The first was that anti-CD25 treatment enhanced antitumor effect (Fig. 1B). The second came from the finding of significant numbers of FoxP3+CD4+ T cells in the liver after LM-D3 treatment. Lymphocytes were harvested from livers and spleens of mice and stained with anti-CD4 and anti-FoxP3 using an intracellular staining technique. Livers from tumor-bearing animals given with LM-D3 treatment had similar percentages and numbers of FoxP3+CD4+ T cells when compared with untreated animals (Fig. 3A and C). Similarly, no major change in this population was seen in the spleen after treatment (Fig. 3B and D).

We therefore sought to determine whether the simple approach of low-dose cyclophosphamide treatment to abrogate Treg activity would enhance the antitumor effects of LM-D3 treatment (21). To set up more stringent treatment conditions, LM-D3 treatment was given to the mice at a later time point after hepatic tumor establishment (the first injection was started on day 5 after tumor challenge). Not surprisingly, LM-D3 treatment was much less effective at treating day 5 tumors than day 3 tumors. A single treatment of cyclophosphamide alone on day 4 showed a minimal effect against hepatic tumors as did LM-D3 treatment alone initiated on day 5. However, mice given with cyclophosphamide and LM-D3 treatment (cyclophosphamide + LM) in this model displayed excellent treatment outcomes (90% survival) compared with the other groups (n = 9–10; P < 0.0001; Fig. 4A). These findings show true synergy between cyclophosphamide and LM-D3 treatment.

To confirm the effect of cyclophosphamide treatment on Treg cells, lymphocytes were harvested from livers and spleens of tumor-bearing mice given with various treatments and stained with anti-CD4 and FoxP3 antibodies. The lymphocytes from the livers treated with cyclophosphamide + LM compared with no treatment or LM-D3 treatment had much fewer FoxP3+CD4+ cells

![Figure 1.](image-url)
on day 14 (0.4% cyclophosphamide + LM, 1.7% no treatment, and 2.2% LM-D3; Fig. 4B). A similar pattern was also found on day 9 (Supplementary Fig. S1). When lymphocytes from the spleen were studied from these same animals, these differences were not found (Fig. 4C; Supplementary Fig. S2).

We also analyzed the total numbers of CD4+, CD8+, and FoxP3+CD4+ T cells found in the livers and spleens of mice given no treatment, cyclophosphamide alone, LM-D3 treatment alone, or both (Fig. 4D). Combination therapy yielded the highest numbers of CD4+ and CD8+ T cells within the liver, which peaked on day 14. Despite higher numbers of CD4+ T cells within the liver on day 14 given with combination therapy, the number of FoxP3+CD4+ T cells remained low. Interestingly, this pattern of increased CD4+ and CD8+ T cells and decreased FoxP3+CD4+ T cells was not seen in the spleen (Supplementary Fig. S3). The ratio of the total numbers of CD8+/FoxP3+CD4+ T cells, which represents the ratio of effector T cells/Treg cells, was highest in the cyclophosphamide + LM group on day 14. The ratios on day 14 were as follows: no treatment, 4.9; LM-D3 (day 5), 6.6; cyclophosphamide, 12.4; and cyclophosphamide + LM, 25.3. This ratio could be an important technique to assess immunologic activity to vaccine therapeutics.

We also studied the presence of antigen-specific CD8+ T cells, as well as their activation status. Lymphocytes from the livers of mice treated with cyclophosphamide + LM were analyzed by flow cytometry using anti-CD8 antibody and Ld tetramer loaded with the immunodominant peptide, AH1. Within the liver, there was a significant increase in the percentage of tumor-specific CD8+ T cells (7.6%) compared with untreated mice (0.6%; Fig. 5A). This pattern was not found in the spleen (data not shown).

Enhanced IFN-γ production and CTL activity with combined cytoxan plus LM-D therapy. To characterize the activity of the CD8+ antigen-specific T cells, lymphocytes isolated from the liver and spleen were stained with anti-CD8 antibody and anti-IFN-γ antibody using an intracellular staining technique (Fig. 5B and C). The highest percentage of CD8+ and IFN-γ+ antigen-specific T cells was found in the liver on day 9 (2.3% cyclophosphamide + LM; 0.7% no treatment; P = 0.016). This difference lessened by day 14 within the liver (1.3% cyclophosphamide + LM and 0.3% no treatment) and was not evident within the spleen (n = 3).

As a whole, these data support that the enhanced effects of the combination of cyclophosphamide and LM are due to (a) control of Treg cells within the liver, (b) increased number of antigen specific CD8+ T cells, and (c) increased activation of these effectors.

We next evaluated cytotoxic activity with an in vivo CTL assay using AH1 peptide (Fig. 6A). In this assay, two populations of differentially CFSE-labeled splenocytes (thus distinguishable on fluorescence-activated cell sorting) are loaded with the antigen-specific peptide or an irrelevant peptide, respectively, and injected i.v. After 36 h, in vivo CTL activity is measured by a diminution in the population loaded with cognate peptide relative to the population loaded with irrelevant peptide. The target lymphocytes were administrated on day 11 after tumor challenge and harvested 36 h later. The group given with combination therapy had 20% killing of targeted cells, whereas the untreated group had 0% (Fig. 6A and B).

Six mice that had survived for 40 days after hepatic tumor challenge and treatment with cyclophosphamide + LM were then rechallenged with subcutaneous tumor as described. Five of the six mice did not have any tumor growth, and one had minimal tumor growth, whereas all of the naive mice (n = 6) had vigorous tumor growth (Fig. 6C). This was highly statistically significant at both 9 and 13 days (P < 0.003 for both).

Discussion

LM is an ubiquitous Gram-positive facultative intracellular bacterium that has been studied as a model for stimulating both innate and T-cell–dependent antibacterial immunity. The ability of LM to effectively stimulate cellular immunity is based on its intracellular life cycle (22). On entry into the host, the bacterium is rapidly taken up by phagocytes into the phagolysosomal compartment. The bacterium is strongly hepatotropic and is readily phagocytosed by Kupffer cells and other phagocytes within the liver, which is the primary site of infection.

Gastrointestinal cancers, such as colorectal cancer and pancreatic cancers, are examples of cancers that occur frequently and tend to form hepatic metastases. Many patients with these cancers die because of disease burden within the liver (4, 23, 24). The ability to control disease within the liver, as well as the ability to induce an immune response to tumor within the liver, has great potential to

Figure 2. Tumor-specific CD8+ T-cell infiltration into the liver. Flow cytometry analysis of infiltrating tumor-specific (AH1) CD8+ T cells into the livers of naive mice or mice that were challenged with tumor on day 0 and underwent either no treatment (NT), a single treatment with LM-D on day 3 (LM-D1), or three treatments with LM-D on days 3, 6, and 9 (LM-D3). Five mice from each group were sacrificed on day 14 to harvest livers for analysis. The only group to exhibit significant increased percentage (A) and numbers (B) of tumor-specific CD8+ T cells into the liver was the LM-D3 mice.
affect survival. Based on the hepatotrophic nature of \( LM \) infection, we have exploited live attenuated \( LM \) as an immunostimulator in the liver. Based on the data from a previous report (5) and this report, a phase I clinical trial for subjects with liver dominant metastatic disease has just opened for accrual at the Johns Hopkins Medical Institutions using live attenuated \( LM \).

We have described previously the use of an engineered live attenuated \( LM \), which homes to the liver following systemic administration, to target antitumor immune responses into the liver for the treatment of hepatic metastases. We showed that this immunorecruitment approach synergizes with a tumor vaccine that produced weak antitumor responses on its own when a single dose was used. We showed that the mechanism of action seems to involve increased activation of local innate effectors, such as NK cells, increased numbers of tumor-specific T cells that are present in the tumor-bearing liver, and increased activity of these cells.

In the previous report, we used a strain of \( LM \) that was attenuated by the deletion of the \( \text{act}A \) gene, which impairs cell to cell spread of the organisms (9). In this report, we use a strain of \( LM \) that is doubly attenuated by the deletion of both the \( \text{act}A \) gene and the \( \text{InlB} \) gene, which promotes entry of the bacterium into certain mammalian cells by binding hepatocyte growth factor receptor (c-met; ref. 8). This doubly attenuated strain of \( LM \), as expected, has a higher \( LD_{50} \) when compared with the singly attenuated strain, but it maintains its ability to elicit an inflammatory immune response in the liver.

In the previous report, we found a strong degree of synergism in a hepatic metastasis model when we vaccinated the mice with the same tumor engineered to secrete GM-CSF 3 days after tumor challenge, followed by treatment with the singly attenuated \( LM \) strain 6 days after tumor challenge. When either the GM-CSF-secreting tumor cell vaccine or a single dose of the singly attenuated \( LM \) strain was given on day 6 alone, these treatments had minimal effect against the hepatic metastases.

In this report, we have concentrated on using the doubly attenuated \( LM \) (\( LM-D \)) strain without any GM-CSF-secreting tumor cell vaccines. We have enhanced the ability of \( LM-D \) treatment to affect the eradication of hepatic metastases alone by

![Figure 3. Decrease in CD4 Treg cells in liver versus spleen (day 14). Flow cytometry analysis of infiltrating CD4+FoxP3+ T cells into the livers of naive mice or mice that were challenged with tumor on day 0 and underwent either no treatment (NT), a single treatment with \( LM-D \) on day 3 (\( LM-D1 \)), or three treatments with \( LM-D \) on days 3, 6, and 9 (\( LM-D3 \)). Five mice from each group were sacrificed on day 10 or day 14 to harvest livers for analysis. The various groups did not have significantly different percentages (A and B) or numbers (C and D) of FoxP3+CD4+ T cells within the liver or spleen on day 14.](https://cancerres.aacrjournals.org/content/canres/67/20/10062/F2.large.jpg)
giving it earlier after tumor challenge (usually day 3 instead of day 6), by giving multiple doses, by combining it with an immunomodulatory agent (cyclophosphamide), or by selectively depleting immune cell subsets.

We show that when multiple doses of $LM$ are used (i.e., three versus one), there is a more marked recruitment of tumor antigen-specific CD8$^+$ T cells to the liver. Treg cells, which are CD4$^+$ and FoxP3$^+$ T cells, have been implicated in the modulation of host immune response.

Figure 4. A, cyclophosphamide enhances $LM$ effect. Survival of mice after tumor challenge on day 0 followed by either no treatment (NT), treatment with 100 mg/kg cyclophosphamide (Cy) on day 4, three treatments of $LM$ on days 5, 8, and 11 ($LM$-D3), or both cyclophosphamide + $LM$ (Cy + $LM$; $n = 9–10$ per group). $P < 0.001$ for cyclophosphamide + $LM$ versus all other groups. B and C, flow cytometry analysis of infiltrating CD4$^+$ FoxP3$^+$ T cells into the livers of naive mice or mice that were challenged with tumor on day 0 and underwent either no treatment (NT), three treatments with $LM$ on days 5, 8, and 11 ($LM$-D3), 100 mg/kg cyclophosphamide (Cy) on day 4, or both (Cy + $LM$). Five mice from each group were sacrificed on day 14 to harvest livers (B) and spleens (C) for analysis. On day 14, the mice receiving cyclophosphamide + $LM$ had the lowest percentage of CD4$^+$ FoxP3$^+$ T cells within the liver but not spleen. D, kinetics of liver-infiltrating cells. Total cells/liver were enumerated on days 9, 14, and 21 for CD4$^+$ T cells in the liver, CD8$^+$ T cells in the liver, and CD4$^+$ FoxP3$^+$ cells in the liver.
immune responses to cancers and cancer immunotherapy (25).

Interestingly, when mice were depleted of CD25+ T cells, the effect of LM-D treatment was markedly improved. When the mice were depleted of CD8+ T cells, the effect of the LM-D was completely abrogated. When the mice were depleted of CD4+ T cells, there was some diminution of the LM-D treatment effect, but this was not significant, suggesting the possibility of a cancelling effect from depletion of Treg cells, as well as T helper cells (which are both CD4+). In aggregate, these data strongly suggest that manipulation of Treg cells can significantly increase immune responses and efficacy of this *Listeria* treatment approach.

When the immunomodulatory agent, cyclophosphamide, was given in combination with LM, there was marked effect in terms of enhancing the survival of mice with hepatic metastases. This effect was so pronounced and unique to the combination, that the first treatment with LM could be delayed until day 5 (instead of the usual day 3) to affect 90% survival of mice that otherwise had 0% survival with no treatment or with either treatment alone. Cyclophosphamide most likely enhances the effect of LM by selectively depleting Treg cells (21). To support this, we showed a substantial decrease in CD4+ FoxP3+ T cells within the livers of mice given with both cyclophosphamide and LM-D treatment. This same diminution of Treg cells was not evident in the spleens of these mice.

When both cyclophosphamide and LM-D treatment were used in combination, (a) the mice had significantly improved survival, (b) there were increased numbers of CD4+ and CD8+ T cells within the liver, (c) there was suppression of Treg cell numbers within the liver, (d) there were increased numbers and percentages of antigen-specific CD8+ T cells within the liver, and (e) these antigen-specific CD8+ T cells were more activated.

We also report some intriguing results of experiments in mice that have a subset of their NK T cell repertoire knocked out (CD1d KO mice; ref. 17). When these CD1d KO mice were used, the ability of the LM-D strain to affect the eradication of hepatic metastases was greatly enhanced. This is perhaps due to the absence of an inhibitory population of NK T cells (18). This finding suggests the possibility that neutralizing interleukin-13 (at least in the liver), which is produced by these CD1d-dependent NK T cells, may have a beneficial effect in the eradication of hepatic metastases in this model (26).

We have not shown direct evidence of Toll-like receptor (TLR) involvement, but TLR2, TLR5, and intracellular innate sensing pathways, including NOD2 and MD5, play an important role in terms of recognition of LM by antigen-presenting cells and this leads to the production of important antitumor (and anti-LM) cytokines (27–33).

The efficacy of LM in eradicating tumor in the hepatic metastasis model is dependent on both the innate and adaptive immune response and requires intact NK and CD8+ T-cell populations. Treatment of LM also decreases the presence of Treg cells within the liver. When these Treg cells are depleted with antibody in mice, the efficacy of LM treatment is greatly enhanced. One way of depleting Treg cells in animals, which is easily translatable to the clinics, is the use of immunomodulatory doses of cyclophosphamide.

![Figure 5](http://www.aacrjournals.org/canres/67/20/10064/fig5.jpg)

**Figure 5.** A, cyclophosphamide + LM increases percentage of tumor-specific CD8+ T cells in liver. Flow cytometry analysis of infiltrating tumor-specific (AH1) CD8+ T cells into the livers of naive mice or mice that were challenged with tumor on day 0 and underwent either no treatment (NT) or treatment with both cyclophosphamide (100 mg/kg) on day 4 and LM-D3 on days 5, 8, and 11 and (Cy + LM). Five mice from each group were sacrificed on day 14 to harvest liver for analysis. Treatment with both cyclophosphamide and LM-D greatly increased the percentage of tumor-specific (AH1) CD8+ T cells. B and C, cyclophosphamide + LM increases activation of tumor-specific CD8+ T cells in liver. Intracellular cytokine staining for IFN-γ by flow cytometry of CD8+ T cells infiltrating the liver (B) or spleen (C) in naive mice or mice that were challenged on tumor on day 0 and underwent either no treatment (NT) or treatment with both cyclophosphamide (100 mg/kg) on day 4 and LM-D3 on days 5, 8, and 11 and (Cy + LM). Three mice from each group were sacrificed on day 9 or day 14 to harvest liver and spleen for analysis. Treatment with both cyclophosphamide and LM-D3 greatly increased presence of intracellular IFN-γ within CD8+ T cells within the liver (B), but not the spleen (C). This effect was more significant on day 9 (P < 0.02 for cyclophosphamide + LM versus no treatment) than on day 14 (P = not significant for cyclophosphamide + LM versus no treatment).
Additionally, a memory T-cell response was shown by the mice that were rechallenged with flank tumor after successfully eliminating hepatic disease following LM-D and cyclophosphamide treatment.

In summary, we have shown an approach to use an organ-specific bacterial infection to target an immune response to a weakly immunogenic tumor withins the liver. This bacterial infection caused by a doubly attenuated LM has the added advantage of having two virulence factors deleted and thus is a more attenuated bacterium to take to the clinics for clinical trial. At present, a phase I clinical trial for cancer subjects with liver-dominant metastatic disease has just opened for accrual at the Johns Hopkins Medical Institutions using attenuated Listeria.

We have also shown in this report that the combined use of cyclophosphamide and LM greatly enhances the eradication of tumor in the hepatic metastasis model. These synergisms result in the influx of tumor antigen-specific T cells that are more highly activated and are more able to do their effector function in vivo.

Perhaps, in addition to genetic attenuation that allows the safe delivery of danger signals within the liver in the context of tumor, LM can be manipulated to deliver specific proteins to the liver such as tumor antigens or cytokines.

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Figure 6. A and B, cyclophosphamide + LM-D3 increases tumor-specific lysis in vivo. In vivo CTL assay in naive mice or mice that were challenged with tumor on day 0 and underwent either no treatment (NT) or treatment with both cyclophosphamide (100 mg/kg) on day 4 and LM-D3 on days 5, 8, and 11 (Cy + LM; n = 3 pooled splenocytes per group). Tumor-specific lysis was much more pronounced in mice treated with both cyclophosphamide and LM-D3. C, six mice that initially survived tumor challenge after cyclophosphamide and LM-D3 treatment were rechallenged in a flank tumor model. Five of six did not have any tumor growth, and one had minimal tumor growth, whereas all of the tumor-challenged naive mice (n = 6) had vigorous tumor growth. Naive mice challenged with tumor were sacrificed on day 14 to spare them discomfort from large tumor volume (*). This was highly statistically significant at both 9 and 13 d (P < 0.003 for both).
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Live Attenuated *Listeria Monocytogenes* Effectively Treats Hepatic Colorectal Cancer Metastases and Is Strongly Enhanced by Depletion of Regulatory T Cells

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